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Proliferative characteristics of the ependymal layer during the early development of the mouse neocortex: a pilot study based on recording the number, location and plane of cleavage of mitotic figures

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INTRODUCTION

In previous studies (Smart 1972*a*, *b*) the proliferative activity of the ependymal layer in the developing spinal cord and diencephalon of the mouse was examined by recording the number, location, and plane of cleavage of mitotic figures at different stages of development. These data were found useful in assessing the productivity of the layer, and in determining the influence of certain limiting factors, which were discussed at some length in the two papers mentioned. In this communication a similar study carried out on the segment of the ependymal layer giving rise to the neocortex is described. This cortical area eventually houses one of the largest cell populations in the mammalian brain and the way the ependymal layer organizes itself to produce these cells shows some interesting modifications of the basic mechanism already described in the spinal cord and diencephalon. The extensive literature on the subject of cortical histogenesis has been summarized by Ariëns-Kappers, Huber & Crosby (1960), and more recently by Berry (1973). The present study is primarily concerned with the activity of the ependymal layer in the very early stages of histogenesis, and supplements rather than overlaps previous work.

MATERIALS AND METHODS

Mouse embryos taken at daily intervals from 10 to 19 days after conception were fixed in Carnoy's solution, embedded in paraffin wax, serially sectioned at 6 μm, and stained with haematoxylin and eosin. At least two embryos at each age period were sectioned in the transverse plane, one in the sagittal plane and one in the horizontal plane. The sets of serial sections in the transverse plane of the 10 to 15 day post-conception specimens were the same as those used in the spinal cord and diencephalon studies referred to in the introduction. The same microscope, lens system, and ocular micrometer were also used, a division of the latter being calibrated at 43 μm. Recordings of the number, location, and plane of cleavage of mitotic figures were made from one set of transverse sections at each age period. The other sets were used for orientation purposes and to make less detailed corroborative counts. The procedures carried out were basically the same as those used in the study of the diencephalon previously reported (Smart, 1972*b*). The recordings were made from sections passing through the caudal part of the cerebral hemisphere, as this area is mainly concerned

with generating neocortical cells and has fewer intrusive features such as the corpus striatum, whose somewhat different pattern of histogenesis dominates more rostral levels.

Surface index

The surface index (the number of mitotic figures occurring per unit length of the surface of the lateral ventricle) at each period was determined by lining up the scale of the ocular micrometer parallel to the surface of the ventricle and recording the number of mitotic figures lying opposite each division of the micrometer scale. Starting from the same reference point in each section the scale was moved progressively along the ventricular wall until a complete circuit had been made. This procedure was carried out in 5 alternate serial sections. The counts from corresponding divisions in each of the 5 sections were summed and then divided by 5 to give the average number of mitotic figures at that particular location. As over 100 divisions were involved the results were simplified by summing together the averages of 5 adjacent divisions and calculating an overall average for each 5 division group, as was done in the diencephalon study (Smart, 1972*b*). The surface indices given in Figs. 1, 3, 9, 11 and 15 thus correspond to the average number of mitotic figures per unit length of the ventricular surface within each 5 division group. The summing procedure was started opposite the hippocampal rudiment and proceeded in a clockwise direction corresponding to the way the diagrams are arranged in the relevant figures. The terminal group usually did not comprise a complete 5 division unit, and the surface index for such incomplete divisions was calculated proportionately. The 5 division grouping corresponded fairly well with the pattern of the counts, and with the exception of the 11 day post-conception specimen did not obscure the transition from one pattern to another.

Recordings of surface mitoses in the neural tube have been made previously by Hamburger (1948) and more recently by Mitolo (1967). Both these authors worked with chick spinal cord. The method of recording used here is basically the same as that devised by Mitolo (1967) whose work was unfortunately unknown to me at the time of my own study of the mouse spinal cord (Smart, 1972*a*).

Area index

The area index, or average number of mitotic figures per unit area of the ependymal layer, was calculated by repeating the procedure for the linear counts, except that this time the number of non-ventricular mitotic figures occurring within the ependymal layer adjacent to each division of the micrometer scale was recorded. These counts were made on the same 5 sections as were used for estimating the corresponding linear index. The results of the surface and deep counts for corresponding divisions in each of the 5 sections examined were then pooled and the total divided by 5 to provide an average. A section from the middle of each series was then projected with a projecting microscope on to paper at a standard magnification (one division of the micrometer scale corresponding to 2 cm on the projection paper), and the outlines of the ventricular surface and ependymal layer were drawn in. The surface of the ventricle was marked off in 2 cm divisions corresponding to those of the micrometer, and the ependymal layer was converted into a series of columns based on these divisions.

The area of each column was estimated using a planimeter. By dividing the area of each column into the average number of mitotic figures occurring within it an estimate of the number of figures per unit area was obtained. The results for every 5 divisions were pooled and averaged as in the linear counts. Area indices were not calculated beyond 15 days of post-conception age, as after that time the boundary of the layer became difficult to distinguish and the packing density of the nuclei decreased and became irregular. The area index is not a very accurate measure of mitotic activity (Smart, 1972*a*), and this was particularly true in the lateral wall of the ventricle, where the periphery of the ependymal layer, except in the early stages, was often ill-defined. Mitotic figures were also particularly numerous in this boundary zone and it was difficult to decide in many cases whether to allocate them to the ependymal layer or not. With these reservations in mind the area index can be used as a basis for forming an opinion, if not a firm assertion, about the total mitotic activity of the layer. The surface index would appear to be a more reliable measure, but even that must be treated with some caution, as the degree of tissue shrinkage varies from fixative to fixative, from species to species, and at different ages of the embryo (Blinkov & Glezer, 1968). These variations introduce another difficulty not only in comparing the findings of different workers but also in interpreting the findings within one species.

Distribution of non-surface mitotic figures

While the counts of non-surface mitotic figures for calculation of the area indices described above were being made, the positions of the figures were estimated and plotted on an outline diagram of the section, as in Figs. 2, 4, 10, 12 and 16. In order to show the distribution of these figures more clearly recordings were made from 5 additional sections, so that in Figs. 2, 4, 10, 12 and 16 the positions of mitotic figures in a total of 10 sections are given. The distribution of these peripheral figures turned out to be a significant feature in the histogenesis of the cortical layer, and for confirmation purposes recordings were made in an additional 10 sections from another embryo at each age period.

Orientations of planes of cleavage

A critical estimate of the plane of cleavage of both surface and non-surface mitotic figures was made by noting the orientation of anaphases and early telophases, the two stages of the mitotic cycle in which the plane of cleavage was least ambiguous. The orientation of 100 consecutive anaphases and telophases was recorded at each surface of the ependymal layer in the 12 to 15 day post-conception specimens. Criteria established in a previous study (Smart, 1970) were used to group orientations into horizontal, vertical, and oblique categories according to the relation of the plane of cleavage to the surface of the ventricle.

Subsurface prophases

Prophase figures occurring among the row of nuclei immediately deep to the nuclei lining the ventricular surface were designated '*subsurface prophases*'. In previous work (Smart, 1972*a*, *b*) their presence was associated with a high surface

index, and they were regarded as belonging to cells which were entering prophase before a vacant space at the apical surface of the epithelium was available. Early prophases were found to be particularly numerous in certain areas of the diencephalon (Smart, 1972*b*) and a search for their presence in the neocortical ependymal layer was also made under a $\times 100$ oil immersion lens. If more than two prophase figures were discovered in each field the area was noted on the outline diagram. The criteria used to distinguish early prophase nuclei were clear nucleoplasm and clumped chromatin lying against the nuclear membrane. In areas where these figures occurred there tended to be a granular appearance of the chromatin in many of the adjacent nuclei (cf. Smart, 1972*a*, fig. 12), suggesting that they were either on the verge of entering a franker stage of prophase, or were late telophases in the last stages of returning to the standard intermitotic nuclear structure. With experience the character of these 'choked' areas became readily recognizable.

Estimation of the change in ventricular area

As an additional aid to visualizing the cephalo-caudal extent of the ventricles and associated cortical areas, atlases of the brain were prepared from a set of serial sections at each age period. Every tenth section was projected with a projection microscope on to paper at a convenient magnification, and the outlines of the brain ventricles, ependymal layer, and other features of histological interest were drawn in. For the 12 and 14 day specimens, the process was repeated on perspex sheets, and the stacked sheets were used to give a three-dimensional picture of the ependymal and cortical layers. The atlases and the perspex models were of value in visualizing the four-dimensional change in the form of the lateral ventricle, and were used in the construction of the outlines of Fig. 21.

Terminology

The terms 'inner' and 'outer' refer respectively to the ventricular and non-ventricular aspects of the neural epithelium. The outer boundary of the ependymal layer is also referred to as its periphery. As the neural epithelium has two surfaces it is possible for structures to be described as 'underlying' either surface. The terms 'dorsal' and 'ventral' refer respectively to the upper and lower poles of the cerebral hemispheres as orientated in the diagrams illustrating this paper. Unless stated otherwise, an age given in days refers to days post-conception. The nomenclature of the various layers follows as far as possible the recommendations of the Boulder Committee (1970). In order to keep the terminology within the present series of papers consistent, however, the terms 'ependymal layer' and 'subependymal layer' are used instead of 'ventricular layer' and 'subventricular layer' recommended by this committee. The term 'subependymal layer' refers to the structure first described by Allen (1912) and a host of later writers, the most recent study being that of Privat & Leblond (1972). Additional features considered important have been described and left unnamed in order not to diversify the terminology further.

RESULTS

10 days post-conception

Histology

The cerebral vesicle at this time was small relative to the size of the interventricular foramen, with the result that no sections of the caudal pole of the vesicle were cut so that they lay on the slide unattached to the diencephalon. The 50 μm thick epithelial wall of the vesicle was composed of 5–6 layers of radially arranged morphologically similar nuclei, and was continuous with the similarly structured epithelium of the diencephalon.

Surface index

The surface index was least (about 0·4) at the mid-line dorsally, and increased to a maximum of about 2·5 at the point of maximum curvature of the lateral wall. Traced ventrally the value declined, but rose again to about 2·6 at the junction of the cerebral vesicle with the diencephalon.

Area index

The area indices of the cerebral vesicle proper paralleled the surface indices.

Mitotic distribution and orientation

Mitotic figures were confined to the ventricular or apical surface of the epithelium, where 95 % of them orientated to give vertical cleavage planes with respect to the surface of the ventricle. A few subsurface prophases were present at the segment of maximum curvature in the lateral wall, where the linear index was maximum.

11 days post-conception

Histology

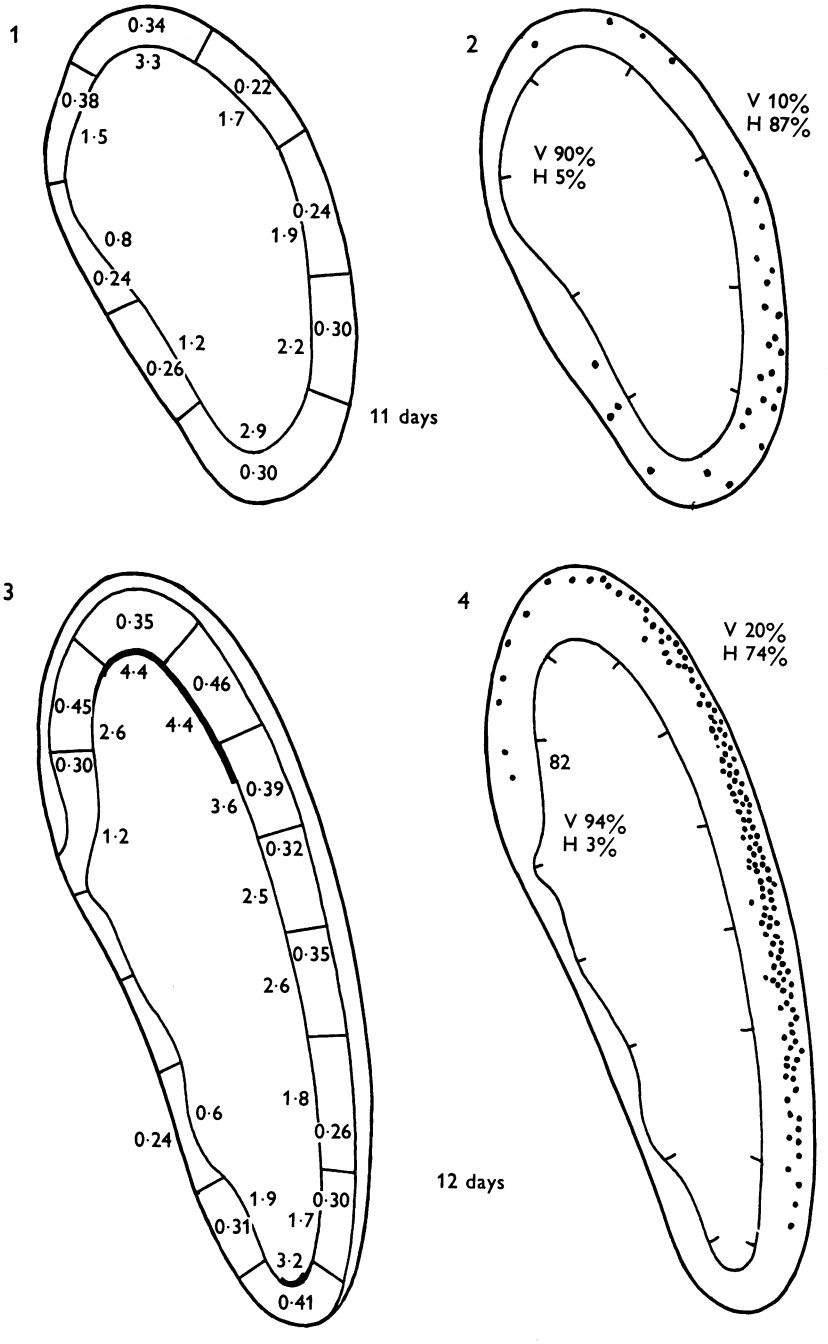
The cerebral vesicle had increased in size and the caudal pole now provided for examination sufficient sections separated from the diencephalon. The epithelium of the vesicular wall had a maximum thickness of about 80 μm and was composed of densely packed, radially arranged nuclei about 8 deep (Fig. 5). At the periphery of the ventrolateral aspect of the vesicular wall there was a layer of rounded nuclei similar to those in Fig. 6. A few capillaries were present in this area. Elsewhere the epithelium was avascular. No marginal layer was present.

Surface index

The average number of mitotic figures per division of the micrometer scale was greatest at the curvatures at the dorsal and ventral poles, where the values for the linear index were 3·3 and 2·9 respectively (Fig. 1). The pooling of the counts from 5 adjacent micrometer divisions did obscure some divisions in both polar regions where the linear index was as high as 3·9.

Area index

The thickness of the epithelium resulted in relatively high values for the area index. This was particularly marked in the dorsal part of the medial wall, where a modest linear index coupled with a thin epithelium gave an area index of 0·38; this was the


1
0·34
3·3
0·38
0·22
1·7
1·5
0·24
1·9
0·8
0·24
0·30
1·2
2·2
0·26
2·9
0·30
11 days
2
V 10%
H 87%
V 90%
H 5%
3
0·35
4·4
0·46
0·45
2·6
4·4
0·30
0·39
3·6
1·2
0·32
2·5
0·35
2·6
1·8
0·6
0·26
0·24
1·9
0·30
1·7
0·31
3·2
0·41
4
V 20%
H 74%
82
V 94%
H 3%
12 days

maximum recorded at this stage (Fig. 1). Elsewhere the area and linear indices paralleled each other.

Mitotic distribution and orientation

Apart from a few peripheral figures, which were most numerous at the ventrolateral aspect of the vesicular wall (Fig. 2), the great majority of mitotic figures lay at the ventricular surface. About 90 % of these surface figures were orientated to give vertical cleavage planes with respect to the surface of the ventricle. *The incidence of vertical cleavage at the ventricular surface remained about this order of magnitude at all subsequent age periods studied.*

12 days post-conception

Histology

Compared to the 11 day specimen the ventricle of the forebrain vesicle at 12 days was larger and the epithelium circumscribing it slightly thicker, particularly the lateral wall, which was about 100 μm across. The epithelium of the upper part of the medial wall and of the upper and lower poles was composed of unmodified, radially arranged nuclei. The lateral wall, however, showed some change in its structure. Its peripheral third was composed of rounded nuclei similar to those distinguished in the subpial region at 11 days, superimposed on an ependymal layer of undiminished thickness (Fig. 6). The rounded nuclei of the outer third were less densely packed than those of the ependymal layer. The distance between nuclei was, however, less than the diameter of one of these round nuclei. This modified area was thickest about the middle of the lateral wall and tapered towards each pole (Fig. 3). Capillaries were present throughout the lateral wall and numerous 3–5 nuclear distances from the ventricular surface.

Linear index

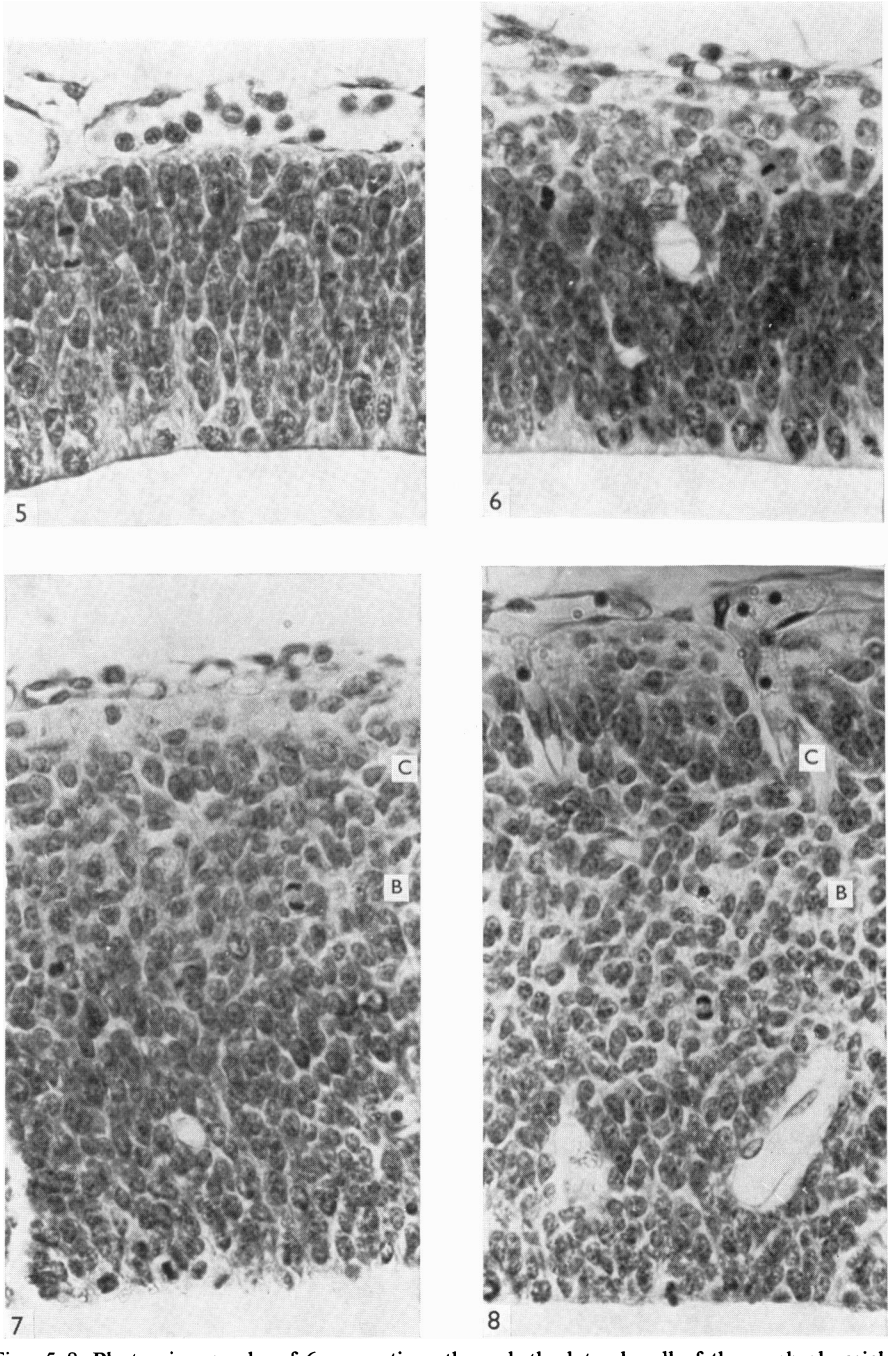
This showed a general increase in value, reaching a maximum of 4·4 in the region of the dorsal pole (Fig. 3). The general pattern was similar to that at 11 days except that the values were proportionately higher.

Fig. 1. Outline of coronal section through caudal pole of cerebral vesicle of 11 day post-conception mouse embryo. The neural epithelium is divided into segments each corresponding to 5 divisions of the ocular micrometer. The figure within the ventricular cavity opposite each box is the surface index or average number of mitotic figures per division of the ocular micrometer corresponding to that segment. The figure inscribed within each box is the area index or average number of mitotic figures per unit area within each box. Dorsal and ventral poles lie above and below, medial and lateral aspects lie to the left and right respectively.

Fig. 2. The same outline as depicted in Fig. 1. The dots correspond to the location of non-surface mitotic figures detected in 10 sections from the same embryo. The boundaries of the compartments in Fig. 1 are marked by ticks on the ventricular surface. The percentages within the ventricle record the percentage of mitotic figures at the ventricular surface which were partitioning vertically (V) and horizontally (H) with respect to that surface. The percentages placed outside the vesicle at the upper right-hand aspect refer similarly to the orientation of the non-surface figures within the vesicular wall.

Fig. 3. Outline of coronal section through caudal pole of cerebral vesicle of 12 day post-conception mouse embryo with surface and area indices marked as in Fig. 1. The thick line marking the ventricular surface at the dorsal and ventral poles denotes areas where subsurface prophases were common. The figure is drawn to the same scale as Fig. 1.

Fig. 4. The same outline as depicted in Fig. 3 showing location of non-surface figures in 10 sections from the same 12 day post-conception mouse embryo. Other features as in Fig. 2.



Figs. 5–8. Photomicrographs of 6 μm sections through the lateral wall of the cerebral vesicle during different stages of its early development. The sections are stained with haematoxylin and eosin and orientated with the ventricular surface below and the basal surface above. Non-surface mitotic figures are circled. All $\times 600$. Fig. 5. 11 day stage. Fig. 6. 12 day stage. Note layer (B) of round more loosely packed nuclei at non-ventricular surface of ependymal layer. Fig. 7. 14 day stage passing through the edge of the cortical plate (C) at periphery of layer (B). The nuclei of the cortical plate are slightly more closely packed than those of the underlying layer (B). Fig. 8. 14 day stage passing through more central part of the cortical plate (C) where the nuclei are slightly larger and tend to be elliptical with long axis radially arranged.

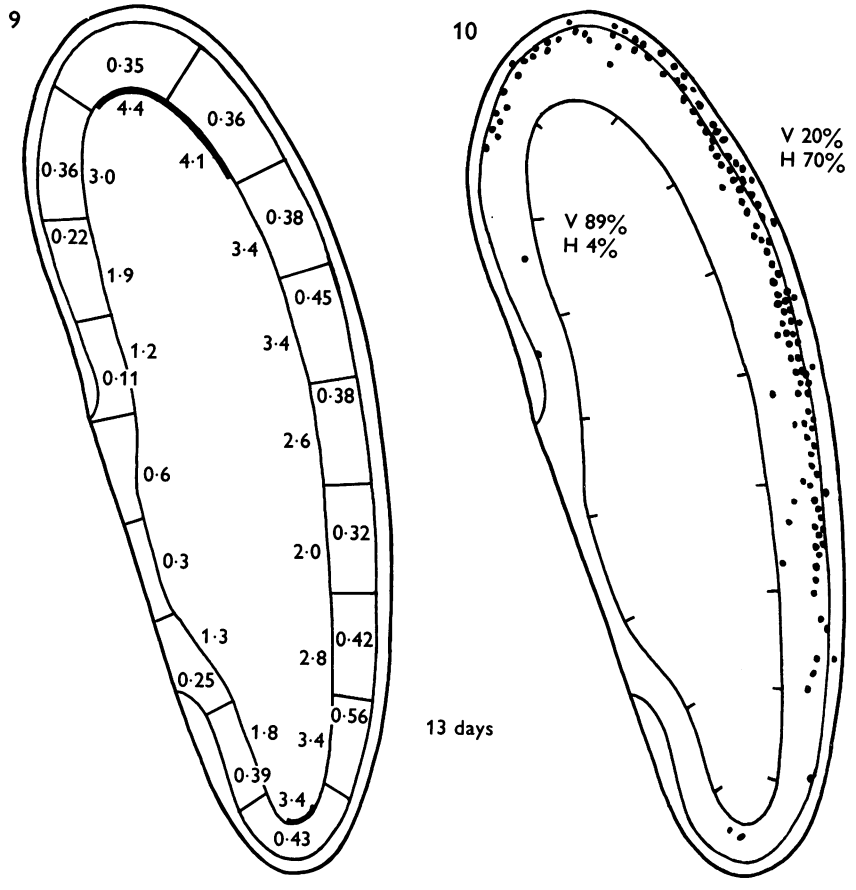


Fig. 9. Outline of coronal section through caudal pole of cerebral vesicle of 13 day post-conception mouse embryo with surface and area indices marked as in Figs. 1 and 3. To the same scale as Fig. 1.

Fig. 10. The same outline as in Fig. 9 showing the location of non-surface figures in 10 sections from the same 13 day post-conception mouse embryo. Other features as in Fig. 2.

Area index

The increased linear index, coupled with the appearance of non-surface figures and a relatively thin epithelium, gave correspondingly high values for the area index, which also paralleled the general pattern found at 11 days (Fig. 3).

Mitotic distribution and orientation

Non-surface mitotic figures appeared in considerable numbers at this age along the lateral wall of the vesicle. They were almost entirely located along the interface between the ependymal layer and the overlying rounded and more dispersed nuclei of the zone of incipient differentiation (Fig. 4); many of them occurred among the nuclei of the zone itself (Fig. 6). These non-surface figures were numerous in the middle region of the lateral wall, where they formed about 20 % of the total number of figures. This distribution was similar in both animals examined for this particular

feature. These peripheral figures had an 80 % incidence of cleavages horizontal with respect to the ventricular surface (Fig. 4). Subsurface prophases were present at both dorsal and ventral curvatures and from the dorsal pole extended down the lateral wall for a short distance (Fig. 3).

13 days post-conception

Histology

At 13 days there had been a further increase in ventricular area and in the thickness of the lateral wall, which reached a maximum of about 120 μm. The increase in thickness was due to the increase in number of more loosely packed rounded nuclei in the peripheral zone; this zone, as at 12 days, was thickest in the middle regions and tapered towards each pole.

Surface index

The values of the linear indices and their pattern of distribution were similar to those found at 12 days, being highest at the dorsal and ventral poles, with a decline towards the middle of the lateral wall (Fig. 9).

Area index

The area indices showed a similar pattern and magnitude to those found at 12 days. The appearance of a substantial number of peripherally located mitotic figures in the lateral wall helped to augment the surface figures and thus maintained a high area index (Fig. 9).

Mitotic distribution and orientation

Non-surface mitotic figures increased in number and remained located along the interface between the radially arranged ependymal nuclei and the peripheral layer of round nuclei. These figures were most numerous in the upper half of the lateral wall (Fig. 10) in both animals examined. In 70 % of cases they were orientated to give a cleavage plane parallel to the ventricular surface.

14 days post-conception

Histology

The increase in the area of the ventricular surface had continued. The ventricular wall had also thickened further, and at the level of sections used in this study the first evidence of cortex formation was evident. This was characterized by a layer of slightly larger nuclei which lay two deep and close together at the outer margin of the layer of more loosely packed round nuclei already described (Fig. 8). Dorsally and ventrally this incipient cortex tapered and blended gradually with the round nuclear layer (Fig. 7). The ependymal layer itself had decreased in thickness to about 80 μm in the area underlying the middle of the cortical zone. Traced through the stacked perspex plates prepared from the 14 day serial sections the contour of this area of incipient cortex had the shape of a fat, rounded comma, as indicated in Fig. 21.

Surface and area indices

The magnitude of both these indices and their pattern of distribution were similar to those found at 13 days (Fig. 11).

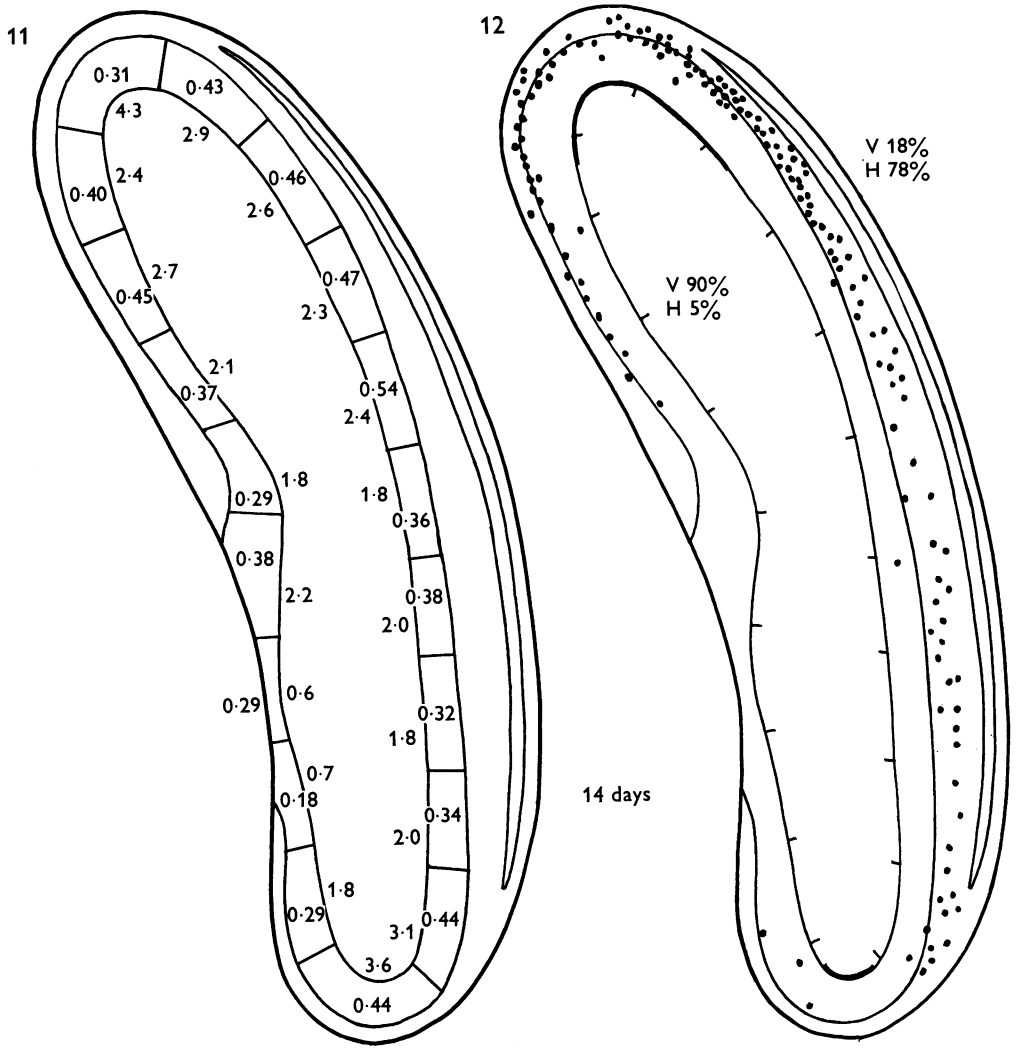


Fig. 11. Outline of coronal section through caudal pole of cerebral vesicle of 14 day post-conception mouse embryo with surface and area indices marked as in Figs. 1, 3 and 9. The scale is the same as in Fig. 1.

Fig. 12. The same outline as in Fig. 11 showing the location of non-surface figures in 10 sections from the same 13 day post-conception mouse embryo as in Fig. 11. Other features as in Fig. 2.

Mitotic distribution and orientation

Non-surface mitotic figures were found scattered along the lateral wall and dorsal part of the medial wall of the forebrain vesicle. They were located mostly at the outer surface of the ependymal layer and were most numerous at the dorsal curvature and under the dorsal part of the incipient cortex (Fig. 12). Deep to the established cortex non-surface mitotic figures tended to be scattered in the layer of rounded nuclei

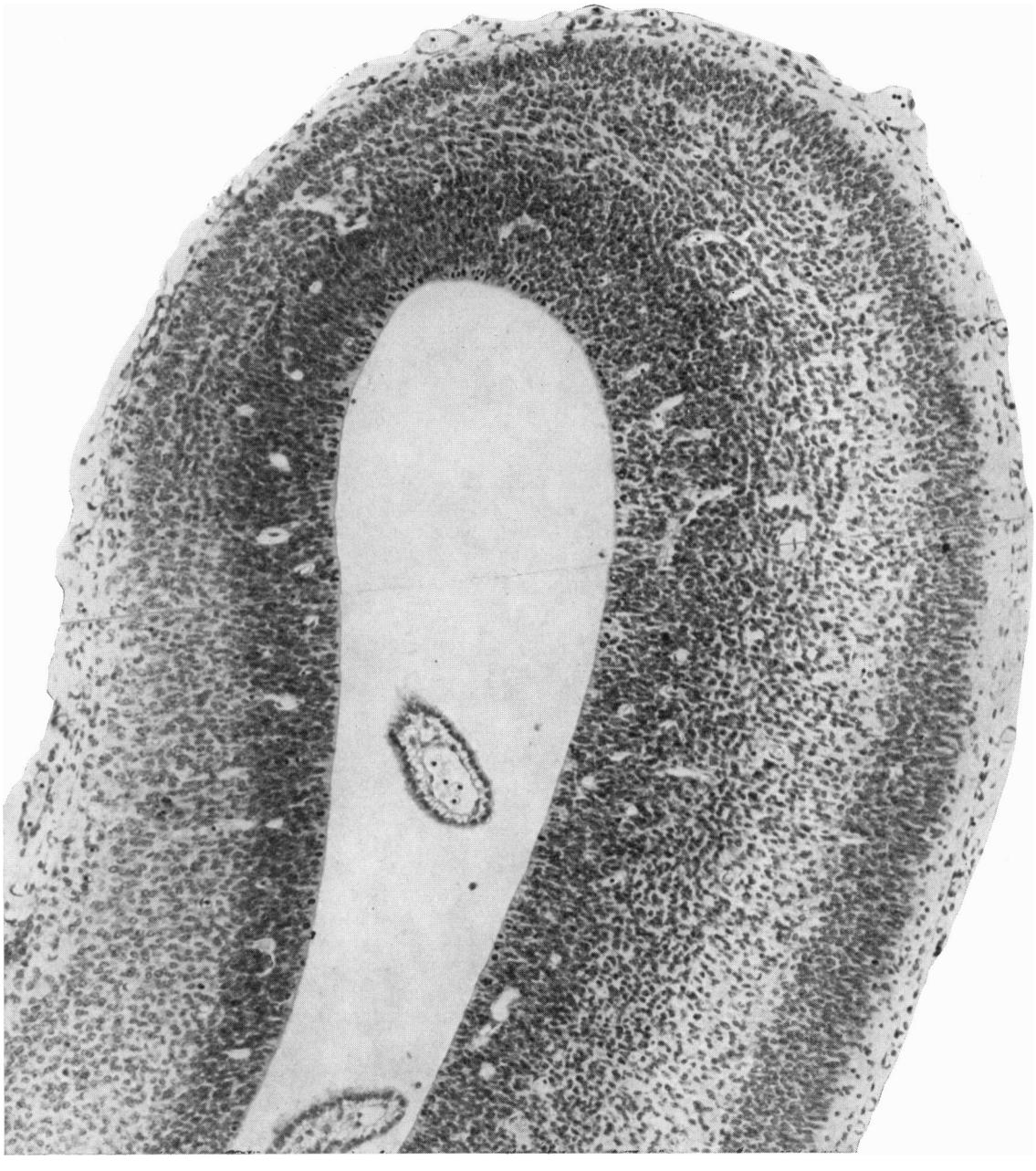


Fig. 13. Montage of two photomicrographs of the dorsal half of the developing cerebral hemisphere of a 15 day post-conception mouse embryo stained with haematoxylin and eosin. The section is one of those used in the counts. Note the density of mitotic figures at the surface of the ventricle at the apical curvature and extending down the medial wall (left) and to a lesser extent down the lateral wall (right). Note the anaphase/teleophase figure (at the top left quadrant) lying at the surface of the ependymal layer under the thin edge of the cortical plate where such figures are most frequent (compare with Fig. 16). *ca.* × 100.

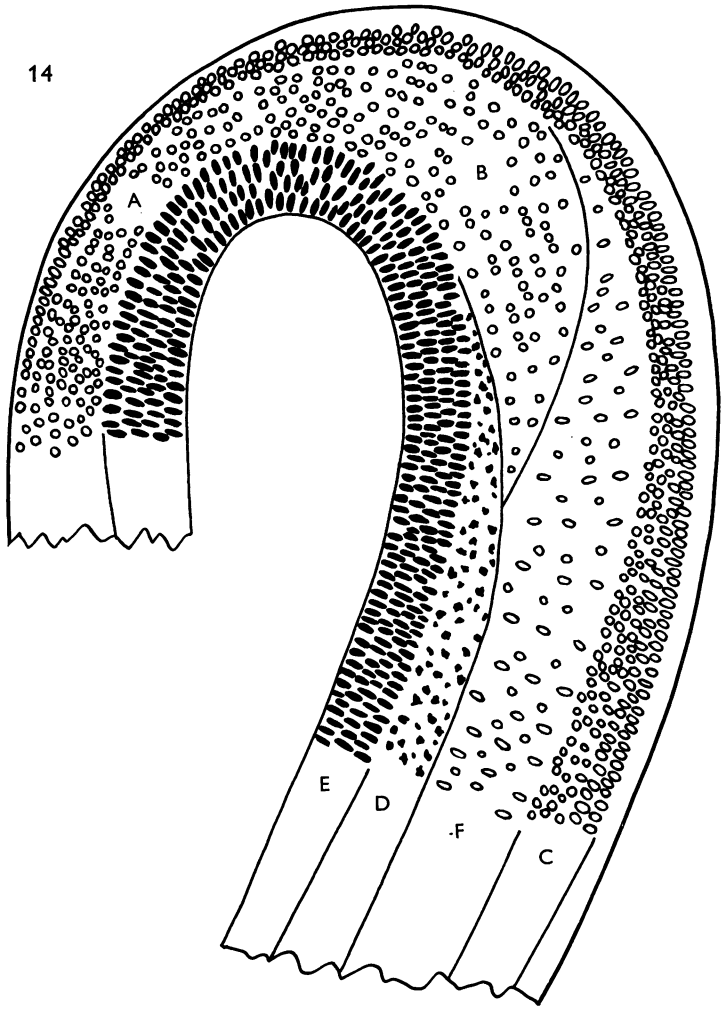


Fig. 14. Diagram traced from Fig. 13 showing different areas of differentiation. E, ependymal layer. A, area of rounded, loosely packed nuclei, the first to differentiate, which merges with area B, composed of similar though more loosely packed nuclei. This in turn merges with area F, composed of rounded nuclei and oval radially arranged nuclei. A, B, and F together form the 'intermediate layer' of the standard descriptions. C represents the cortical plate. Note how it tapers when traced anticlockwise. D represents the irregularly orientated polymorphic nuclei of the subependymal layer.

(Fig. 7). This pattern of distribution was present in both animals examined for this feature. The majority (78 %) were cleaving in a plane parallel to the ventricular surface.

15 days post-conception

Histology

In the 15 day specimen the main histologically distinct developmental areas had been established. These are depicted in the photomicrograph in Fig. 13 and the corresponding diagram in Fig. 14. The ependymal layer retained the character and thick-

ness found at 14 days. The layer of more loosely packed round nuclei had moved to the medial side of the dorsal pole of the section, extending laterally in a quadrant from about 9 o'clock to 12 o'clock (Figs. 13, 14, area A). Traced laterally the nuclei of this area became more dispersed (Figs. 13, 14, area B) and were either elliptical, with radially arranged long axes, or rounded, with a tendency to form circumferential rows. Further laterally still the nuclei of this zone become even more widely separated (Figs. 13, 14, area F). Areas A, B and F together correspond to the 'intermediate zone' of the standard descriptions. At the outer surface of the ependymal layer related to the lateral wall of the ventricle, a layer of smaller irregularly shaped nuclei had appeared (Figs. 13, 14, area D); these were different in character from the rounded nuclei of area A or B. Laterally area D blended with the overlying area F. Dorsally and ventrally it tapered to blend with the ependymal layer and area B. Traced cephalad it was continuous with a similar aggregation of cells involved with generating the cell populations of the corpus striatum. This layer appeared to be the predecessor of the subependymal layer. The developing cortex (Figs. 13, 14, area C) extended in an arc from the medial wall, where it was about one cell thick, to the lateral wall, where it was about 10 cells thick (Fig. 17). It gained its maximum thickness approximately opposite the end of area B (Figs. 13, 14). Where the cortex was thin it was composed of rounded or moderately oblong nuclei as in Fig. 8, but where it had achieved its maximum thickness the inner part was composed of nuclei of this character, while the nuclei of the outer part were elliptical or pear-shaped (Fig. 18).

Surface and area indices

The magnitude and pattern of distribution of these indices remained similar to the situation at 14 days (Fig. 15).

Mitotic distribution and orientation

Mitotic figures were most numerous in the region under the most newly formed part of the cortical layer, where they lay at the periphery of the ependymal layer. Elsewhere they were found dispersed among the scattered nuclei of the subcortical cells, particularly in the subependymal layer (Fig. 16 and Figs. 14, 15, area D). The orientation of peripheral figures continued to give a majority (72 %) of horizontal cleavage planes.

16 days post-conception

Histology

The ependymal layer had diminished in thickness throughout the entire length of the lateral wall, where it was composed of 2–3 layers of more rounded nuclei which blended with the subependymal layer of polymorphous, irregularly arranged, loosely packed nuclei (Fig. 19). At the dorsal pole and dorsal part of the medial wall the ependymal layer retained its original maximum thickness, with closely packed radially arranged ellipsoidal nuclei. Thickening of the cortical plate had spread dorsally and ventrally.

Surface index

The index retained a value of about 3·0 in the region of undiminished ependymal thickness, but elsewhere it declined to around 1·0.

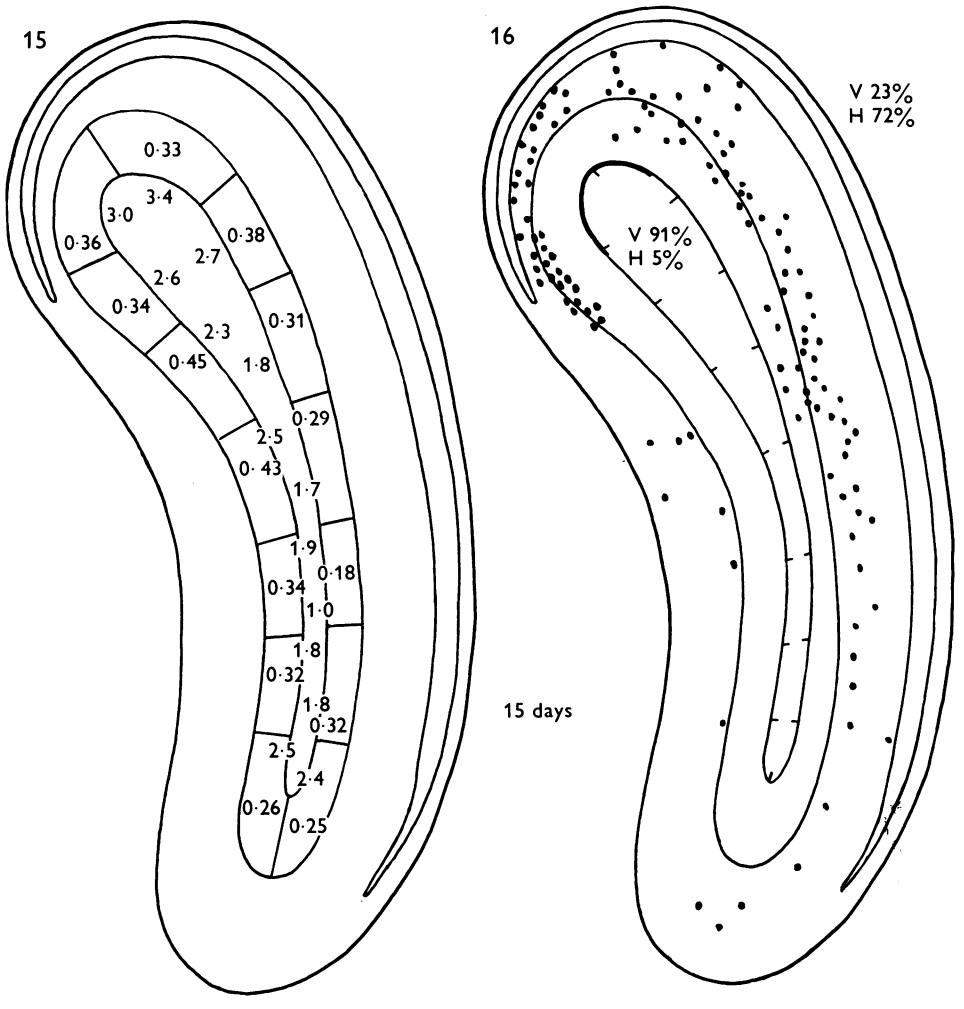


Fig. 15. Outline of coronal section through caudal pole of cerebral hemisphere of 15 day post-conception mouse embryo with surface and area indices marked as in Figs. 1, 3, 9 and 11. The scale is the same as in Fig. 1.

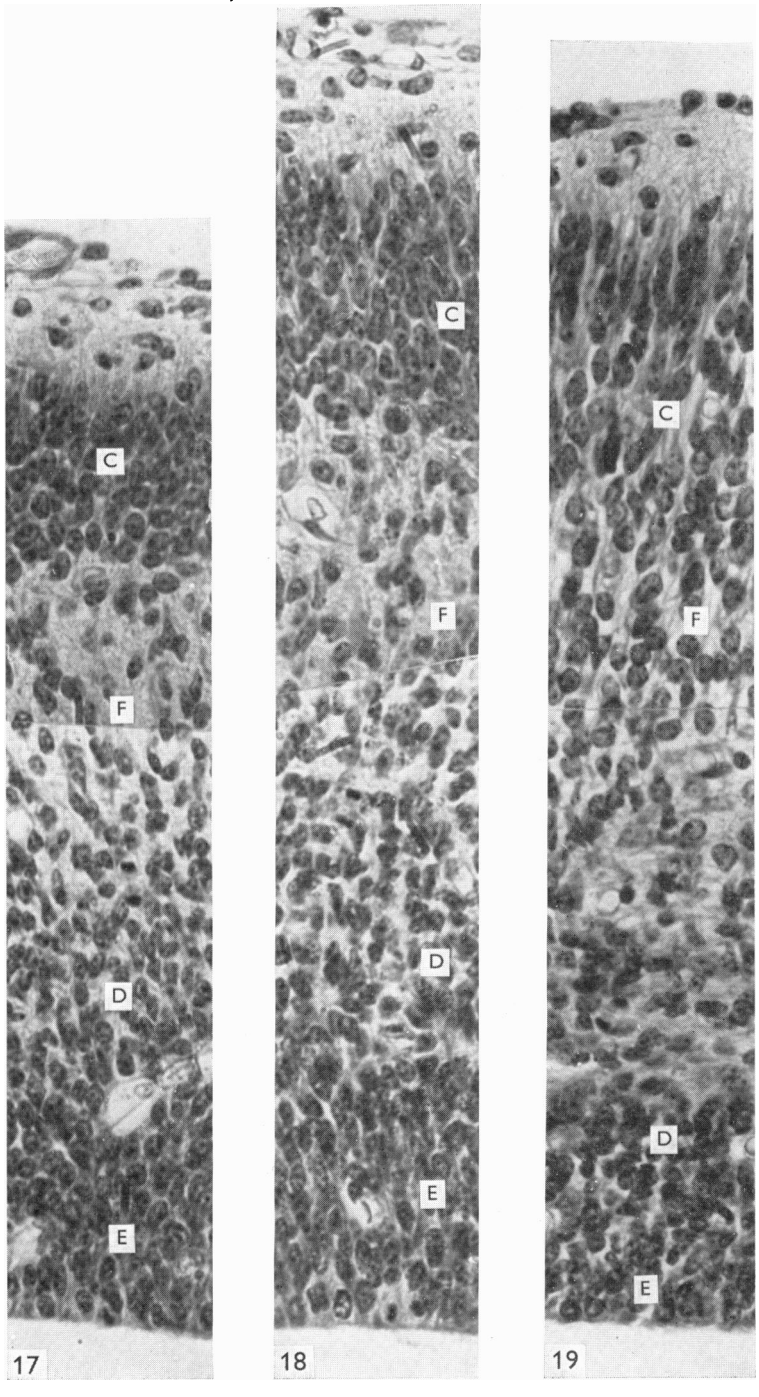
Fig. 16. The same outline as in Fig. 15 showing the location of non-surface figures in 10 sections from the same 15 day post-conception mouse embryo as in Fig. 16. Other features as in Fig. 2.

Area index

The difficulty in defining the periphery of the ependymal layer and the change in packing density of the nuclei of the layer from 16 days onward made calculation of the area index impracticable.

Mitotic distribution and orientation

As before, the non-surface mitotic figures were most numerous at the periphery of the ependymal layer underlying the most newly formed cortical area. Elsewhere they were scattered between the ependymal layer and the overlying cortex.


C
F
D
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17
C
F
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18
C
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19

17, 18 and 19 days post-conception

Histology

During the last days of prenatal development the ependymal layer regressed progressively along the entire ventricular wall until it was entirely composed of a single layer of cuboidal cells. The subependymal layer similarly regressed but remained several layers thick at the dorsal pole and housed mitotic figures throughout its extent. The cortical plate appeared to reach its maximum extent by 18 days.

Linear index

This decreased progressively towards the dorsal pole, and by 19 days had a value of less than 1·0.

Mitotic distribution and orientation

Non-surface mitotic figures were found in the subependymal layer and in the intermediate layer intervening between it and the cortical plate. Their orientation was irregular.

Change in area of ventricular surface

As far as could be judged from the atlases the ventricular surface associated with the neocortical area increased throughout the prenatal period. The increase, which was more rapid up to 14 days and thereafter slowed down, appeared to be greater in the long axis than in the transverse plane. This was associated with the growth of the olfactory ventricle and occipital pole of the cerebral hemisphere proper.

DISCUSSION

The level of the sections chosen for study sample more than the neocortical portion of the telencephalic vesicle. Parts of the developing hippocampus, amygdala, and pyriform cortex appear in most sections and blend with the neopallial component. Although counts were made on the ependymal layer underlying all these areas, comment will be restricted to the segment of the layer concerned with production of the neocortex. If sections of the developing spinal cord, diencephalon, and neocortical component of the telencephalon are set side by side, as in Fig. 20, a certain similarity in their pattern of development may be observed. In the studies on the spinal cord (Smart, 1972*a*) and diencephalon (Smart, 1972*b*) it was found convenient to divide the proliferative activity of the ependymal layer into three stages (which, I have

Figs. 17–19. Photomicrographs of 6 μm sections through the lateral wall of the cerebral vesicle of developing mouse brain. The sections are stained with haematoxylin and eosin and orientated with the ventricular surface above and the basal surface below. Non-surface mitotic figures are circled. All sections $\times 600$ as in Figs. 5–8 of the present publication and Figs. 4–6 in Smart (1972*b*). Fig. 17. 15 day stage passing through a thinner area of cortical plate. Note all cortical nuclei are rounded or short ellipsoids. Fig. 18. 15 day stage passing through thicker area of cortical plate Outer cortical nuclei tend to be elongated. Letters E, D, F and C denote the same areas as in Fig. 14. Fig. 19. 16 day stage. Ependymal layer, E, has regressed and has been replaced by a layer of smaller polymorphic nuclei which seem to be the precursors of the subependymal layer of the adult. Cortical nuclei are less densely packed and are composed of an outer zone of elongated and an inner zone of rounded nuclei.

since discovered, correspond substantially to the concept of *Matrixphasen* proposed by Kahle, 1951); Stage 1 in which proliferation occurs without differentiation; stage 2 in which differentiation balances proliferation; and stage 3 in which differentiation exceeds proliferation and the proliferative compartment undergoes decline. A similar progression is found in the developmental pattern of the neocortical area (Fig. 20). The neocortical stage 1 and 2 compartments, however, are curved as in Fig. 21, appearing twice in most sections (compare left and right-hand sides of Fig. 21), and surround a central stage 3 compartment. In terms of cell production the slower the progression through this sequence the greater will be the final product of cells (see discussion in Smart 1972*a*). The period spent in the generation of a pure stage 1

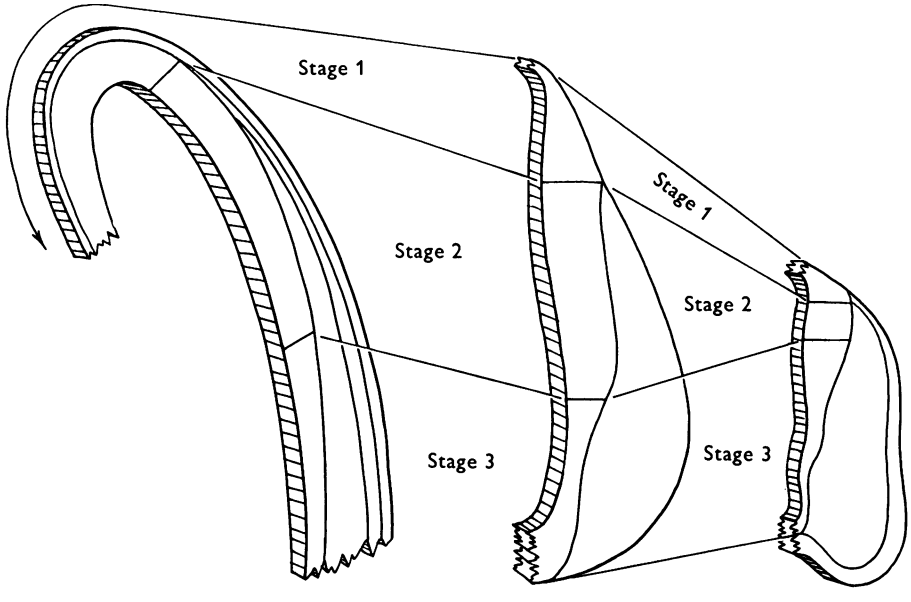

Fig. 20. Outline drawings of sections of developing cerebral vesicle, diencephalon and spinal cord from left to right respectively to show in each case the relative sizes of the three proliferative compartments defined in the text. The cerebral vesicle section corresponds to the appearance at 14 days post-conception and the other sections to the appearance at 13 days (compare Smart, 1972*b*, fig. 11). The scale of the drawings is the same.

compartment is about 3 days (from 10 to 12 days of post-conception age, taking into account that the peripheral rounded nuclei at 12 days seem to be part of the proliferative compartment), which is longer than in the spinal cord (1 day, Smart 1972*a*) and diencephalon (2 days, Smart 1972*b*). The stage 2 compartment appears about 13 days of post-conception age at the lower part of the lateral wall and extends progressively upwards following the retreating boundary of the actively proliferating stage 1 compartment. The decline of the ependymal layer in this area into stage 3 begins about 14 days. In the spinal cord and diencephalon stage 3 terminates by the fairly rapid transformation of the ependymal layer into a single layer of cells. This is accompanied by a decrease in the area of the ependymal surface, either as a result of reduction in size of the central canal as in the spinal cord, or because of fusion of opposite walls of the

third ventricle as in the diencephalon. In the cerebral vesicles, however, the stage 3 compartment can be considered to end when the nuclei of the cells of the ependymal layer lose their early characteristic closely packed radial arrangement to become (with the exception of the surface layer which forms the ependymal lining of the ventricle) polymorphous, irregularly orientated, and more loosely packed, as in Fig. 19. This polymorphic nuclear population contains frequent mitotic figures, and is continuous with a similar cell population which underlies the developing corpus striatum and olfactory cortex. It is the forerunner of the much discussed subependymal layer or

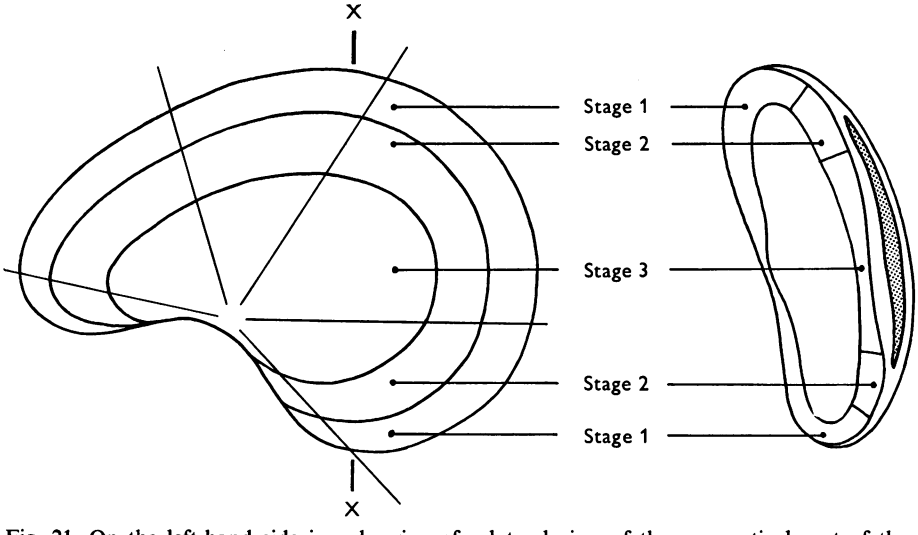

Fig. 21. On the left-hand side is a drawing of a lateral view of the neo-cortical part of the cerebral vesicle extrapolated from serial sections of the brain of a mouse embryo of about 14 days of post-conception age, showing the shape of the three proliferative compartments described in the text. The notch in the antero-lateral aspect of the diagram marks the boundary with the pyriform cortex (which has been omitted from the diagram as this communication does not deal with the histogenesis of this area.) On the right is depicted a cross-section taken through the lateral view at level marked X–X. The left-hand diagram can also be used to depict the growth of the neocortex. The three concentric outlines correspond to the shape and relative sizes of the cerebral hemispheres at 12, 13 and 14 days of post-conception age respectively. The 5 lines radiating from the centre of similitude or 'umbo' of the system relate to the commentary on cortical growth which concludes the discussion.

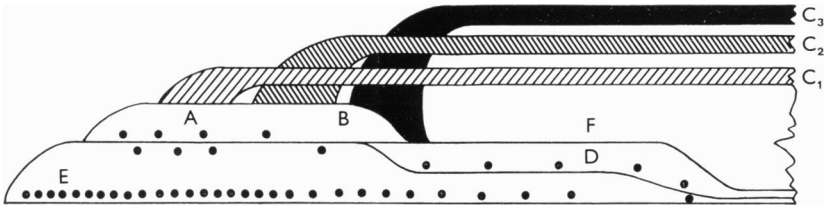

Fig. 22. Diagram (modified from Berry & Rogers, 1965) representing a model for the growth of the neocortex. The letters A–F relate to the same areas as in Figs. 13 and 14. C_1, C_2, C_3 refer to the cortical cell population laid down at successive times as described by Berry & Rogers (1965). The black dots indicate the relative density of mitotic figures. The model is to be visualized as moving to the left as the ependymal layer at E advances by proliferation, followed by the other progressively maturing areas.

plate, the cells of which continue to proliferate during adult life. Consideration of the natural history of this interesting tissue thus extends beyond the present area of study and will not be discussed here. The proliferative events within each compartment are similar to those found in the diencephalon, albeit with certain modifications. In stage 1 the linear index reaches its maximum value of around 4·4, which seems to represent the maximum *average* number of mitotic figures which can be accommodated by 43 μm of ventricular surface. This proliferative activity leads to increase in area of the ventricle rather than to increase in pseudostratification. The high linear index, together with the low degree of pseudostratification, thus produces high area indices and suggests a high mitotic rate. The complications in assessing the true rate of cell production introduced by possible variations in the length of the generative cycle have been discussed previously (Smart 1972*a*, *b*). The neocortical stage 2 compartment has similar characteristics of the corresponding diencephalic compartment – a slight reduction in linear index compared to stage 1 and the presence of non-surface mitoses, the two sets of figures combining to give an area index not much lower than in the stage 1 segment. In stage 3 the surface index declines to very low values, and although non-surface mitotic figures are present the change in density of nuclear packing makes the calculation of the area index valueless.

In the initial paper of this series (Smart, 1972*a*), two 'rules' were proposed as heuristic guides. The first stated that in order to produce the maximum number of nerve cells in a given number of generations differentiation must be delayed. The second rule stated that given the pseudostratified structure of the ependymal layer, accumulation of ependymal cells will be limited by saturation of the apical surface of the epithelium by mitotic figures unless there is a corresponding increase in the area of the central canal, or a modification of the pseudostratified structure allowing dispersal of mitotic figures. The rules governing cell production by the neural epithelium are thus somewhat similar to those governing other capital investments. The longer capital remains invested in the bank at compound interest the more it earns; once it is converted into cash, i.e. turned into neurons, it ceases to earn interest (rule one). As the interest accumulates in the vault of the bank the space it occupies will increase and eventually fill the vault; if the regulations of the bank require the units of capital to be shifted about from one side of the vault to the other as interest is earned, then this to and fro traffic will gradually become more and more congested as the vault fills up (rule two). The situation can be improved by building a narrow vault to minimize the distance between the walls and thus reduce the distance the units of capital have to be shifted to acquire each increment of interest. Provided the walls remained close together, the vault could increase in length and height as interest accumulated. Alternatively, the regulation requiring the shifting of the units of capital from one side of the vault to the other could be suspended. In the neocortex the operation of the first rule is seen both in the longer period of pure proliferation without differentiation and in the slower progress through the stages compared to similar events in the spinal cord or diencephalon. The growth of the thin-walled cerebral ventricle in early development can be interpreted as a means of banking mitotic (i.e. interest-earning) precursor cells in a narrow vault prior to converting them to non-dividing neurons. Although the low degree of pseudostratification in the cerebral vesicles produces the recommended narrow vault, it does not

appear to be narrow enough in relation to the rate of movement of the units of capital to prevent congestion from occurring. The operation of the second rule is thus suggested by the presence of subsurface prophases between 12 and 14 days in those areas where the surface index is high. The appearance of additional peripheral figures in all stages of mitosis suggests the gradual abolition of the migration regulation.

The location of these non-surface mitotic figures is interesting. Subsurface prophases were only found in any quantity at the dorsal and ventral curvatures and the dorsal part of the lateral wall; their presence in these situations coincided with high values for the surface index, and can be reasonably interpreted as the result of migrating nuclei entering mitosis before a surface vacancy has become available. Virtually all other non-surface figures were restricted to the peripheral regions of the ependymal layer. At 12 and 13 days such figures were present in large numbers in the lateral wall subjacent to the area where the cortical plate would make its initial appearance (compare Figs. 10 and 12). Later they were most numerous underlying the thin leading edge of the cortical plate and in the adjacent fringe of the round nuclear layer (Fig. 12). Beneath the established cortical plate non-surface figures were less numerous, and occurred either in the subependymal layer or along the interface between it and the ependymal layer. The polarization of mitotic figures to the inner and outer surfaces of the neocortical endymal layer was more marked than in the diencephalic region. In the former situation over 90 % of the non-surface figures lay at the outer boundary, whereas in the thalamus less than 50 % lay at the periphery of the layer and the remainder were scattered throughout its substance. Another interesting difference between the two areas is that non-surface figures were much more numerous in the thalamic ependymal layer, where at times they exceeded in number the corresponding surface figures (Smart, 1972*b*). In the neocortical ependymal layer, on the other hand, the major site of mitotic activity during cortical histogenesis remained at the ventricular surface, where some 80 % of the total number of figures were located. The location of these non-surface figures predominantly at the outer boundary of the ependymal layer and their predilection for horizontal cleavage indicate the operation of some controlling mechanism. The scanty but interesting literature describing the precision with which cells can orientate their mitotic figures was discussed in a previous communication (Smart, 1970), and recent work on the possible mechanisms controlling migration of nuclei has been reviewed by Berry (1973). In neither case is there any clear evidence indicating the nature of the mechanisms controlling these movements.

In the previous communications in this series (Smart, 1972*a*, *b*) pains have been taken to regard the two rules as heuristic devices, that is rules made with a view to learning something from their application and not as moulds into which the facts are to be fitted. The evolution of the second non-surface population of figures is unlikely to be a simple response to surface congestion as the second rule, baldly stated, might suggest. Although the initial appearance of non-surface figures in any quantity seems to be generally associated with areas where the surface index is high and where subsurface prophases are present, the distribution of non-surface figures within the ependymal layer is not what would be expected from passive congestion. Mere lack of room at the surface is more likely to produce accumulations of dividing cells in all

stages of mitosis in the immediate subsurface nuclear layers – accumulations similar to the striking metaphase crowding produced by blockading mitosis by colchicine (Watterson, 1965). Also, non-surface mitotic figures occur in the lateral wall of the ventricle under the established cortex, where the surface index is low and subsurface prophases are uncommon (Figs. 15, 16). This suggests that, once evolved, the proliferative activity of the non-surface population is independent of the proliferative activity of the surface migrating cells. At 11 days, too, a small population of peripheral figures is present before surface mitotic activity has reached its maximum (Figs. 1, 2 and 5), which suggests that the evolution of the peripheral mitotic population may be initiated before surface saturation has been achieved. Here it must be added that the presence of non-ventricular mitotic figures in the developing central nervous system is no new discovery. The existence of such figures was the subject of some controversy among workers at the end of the last century. Altmann in 1881 stated that during embryonic life all epithelial organs, including the brain, grew by cell proliferation occurring at the surface of the epithelium which was furthest away from the mesoderm. Rauber (1882), for theoretical reasons and from a certain amount of observation, declared otherwise. Merk (1885) published a long paper describing the distribution of non-surface mitotic figures in different areas of the developing brain of a wide variety of vertebrate species, and his results confirmed Rauber's opinions. He described, for example, non-ventricular figures occurring in the cerebral vesicle in a 2–3 mm mouse embryo and performed a count of the number of surface and non-surface mitotic figures in 25 sections of the cerebral vesicle. In pooled counts he found a ratio of 50:1 between ventricular and non-ventricular figures. Unfortunately, the age of the embryo and the level at which the counts were made are not clear from Merk's description, and no comparison can be made between his somewhat lower frequency of non-surface figures and the findings of the present study.

Although we are concerned primarily here with the proliferative characteristics of the ependymal layer, some interesting deductions can be made about the formation of the cortical plate by following changes in its extent, relative thickness, and relation to sites of mitotic activity at successive stages of development. The ependymal layer in its early stages is composed of closely packed oval nuclei arranged with their long axes more or less at right angles to the ventricular surface (Fig. 5). The uniform radial arrangement of ependymal nuclei first breaks down along the lateral wall of the cerebral vesicle. The most peripheral nuclei become rounder and less densely packed but at first show no tendency to condense into a cortical plate (Fig. 6). Such rounded nuclei are found, although in small numbers, at the subpial aspect of the epithelium as early as 11 days. By 13 days they occupy about one-third of the thickness of the lateral wall of the cerebral vesicle. Traced in three dimensions this area has the shape of a fat comma with its short blunt tail directed cephalad (Fig. 21). This arrangement is characteristic of the first non-ependymal cells formed from the stage 2 compartment, and these cells are found appearing in the zone where mitotic activity at the periphery of the ependymal layer is at its maximum (Figs. 4, 10). It seems probable that some at least of this population of nuclei are recruited from the daughter cells of the mitotic figures found at the periphery of the ependymal layer. The majority may have this origin, as surface mitosis in the early stages of development is probably largely contributing to the increase in area of the ventricular surface.

According to Berry (1973) these peripheral, round nucleated cells may be referred to as neuroblasts because it can be shown autoradiographically that they later differentiate into neurons. At 14 days the first evidence of cortex formation appears in the form of a distinct layer of closely packed nuclei at the outer boundary of the zone of loosely packed rounded nuclei (Figs. 7, 8 and 12) in the region of the middle of the lateral wall of the cerebral vesicle. The cortical plate is thickest centrally (Fig. 8). Traced towards its circumference this thin lamina of nuclei tapers gradually (Fig. 7) until it blends with the layer of rounded loosely packed nuclei, which in turn blends with the outer surface of the ependymal layer. In three dimensions these three layers have the form of fat commas of diminishing size set one within the other, as in Fig. 21. By 15 days, a considerable increase in the thickness of the central part of the cortical plate has occurred and the whole plate has increased in area. Mitotic activity at both surfaces of the ependymal layer is greatest in the area around and under the thin leading edge of the cortical plate (Figs, 13, 15 and 16). Beneath the established cortex at 15 days both the surface index (Fig. 15) and the number of non-surface figures (Fig. 16) decline, but as the ependymal layer also decreases in depth the area index remains fairly high, at around 0·30 (Fig. 15). After 15 days, regression of the ependymal layer in thickness and mitotic activity procedes centrifugally (from a centre of similitude in the lateral wall, Fig. 21) until the histogenetic process goes to completion.

According to the autoradiographic studies of Angevine & Sidman (1961), and particularly the more recent studies of Berry & Rogers (1965), the layers of the cortex are laid down by successive migrations of cells which pass through each other, so that the first migration forms the deepest layer of the cortex and the last the most superficial. It is implied by Berry & Rogers (1965) that each migratory wave establishes a cortical layer simultaneously throughout its extent, so that it is possible to say, for example, that in the rat the cells of cortical layer V are formed from the ependymal layer on the 17th day, those of layer IV on the 18th day and so on. It seems evident from the present study that, in the mouse at least, a given cortical layer is not laid down over its entire ultimate extent by a single simultaneous migration from the ependymal layer, but that the cortex is built up by addition of cells in all layers over a period of days, and matures along gradients which radiate from the central earliest maturing compartment, as depicted in Fig. 21. (At 15 days, as we have seen in Figs. 13 and 14, the cortex at the middle of the lateral wall already consists of cells which could be candidates for most cortical layers, whereas at the dorsal pole only the initial round nucleated cells are present.) This situation can be reconciled with the views of Berry & Rogers if the diagram used to illustrate their findings (Berry & Rogers, 1965, Fig. 8) is thought of as adding to the cortex by moving to the left. In Fig. 22 the changes in histological appearances and mitotic distribution derived from the present study are assembled into a linear sequence representing a profile taken through any of the radial lines in Fig. 21. It is based on and complements Fig. 8 of Berry & Rogers (1965). The suggested histogenetic process would proceed as follows. Maximum ependymal proliferation without differentiation (stage 1) occurs along a curved zone at the sagittal circumference of the cerebral hemisphere, as depicted in Fig. 21. As ependymal proliferation leads to increase in circumference, the central area left behind by this advance commences to shed cells derived partly or

wholly from peripheral mitoses, as in Fig. 6, and the ependymal layer enters stage 2. These cells presumably form the deeper cortical layers. Then, as the section of ependymal layer we are following is left progressively further behind by further circumferential advance, it sheds further neurons, apparently derived mainly from the population of ependymal cells whose nuclei migrate to and from the ventricular surface. These later formed neurons would migrate through the cortical neurons already formed, as described by Berry & Rogers (1965), so that the more superficial the layer of the cortex, the further from the advancing sagittal circumference will be the segment of the ependymal layer from which it originates (Fig. 22). Such a 'shifting distribution' was described in the rat by Hicks & D'Amato (1968) who found, for example, that ependymal cells labelled with ^{3}H-thymidine at 16 days post-conception became located in the adult in layers II and III of the lateral isocortex and in layers V and VI of the frontal, dorsal and occipital areas of the isocortex. Later, as established cortical cells commence to mature, ependymal and particularly subependymal mitotic activity in areas distant from the sagittal circumference can be expected to be increasingly concerned with supplying the neurons and their growing processes with the many times larger population of neuroglial cells with which neuron growth and metabolism are so closely linked. The changes in the histological character of the intermediate layer, as traced clockwise through areas A, B and F in Figs. 13 and 14, and the continuing proliferative activity of the subependymal layer would reflect this change-over.

SUMMARY

Cell production by the ependymal layer in the telencephalon of mouse embryos 10–19 days after conception was studied by recording the number, location and plane of cleavage of mitotic figures. The pattern of proliferative activity in the neocortical segment of the telencephalon showed interesting similarities to the basic pattern previously described in the spinal cord and diencephalon. The most active site of cell production in the telencephalon, as judged by the number of mitotic figures per unit length of the ventricular surface, lay along the sagittal circumference of the telencephalic vesicle beyond the margins of the developing cortical plate. Mitotic figures lying away from the ventricular surface were most numerous in the immediate vicinity of the margin of the cortical plate. The majority of these non-surface figures were located at the interface between the ependymal layer and the incipient cortical plate. Of these figures 70–80 % were orientated to give horizontal cleavage with respect to the ventricular surface. It appeared that the earliest formed cells of the cortical plate probably originated from daughter cells of these peripheral mitoses. These findings are discussed in relation to the current hypotheses on cortical histogenesis.

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